

Rac1-induced cell migration requires membrane recruitment of the nuclear oncogene SET

Jean Paul ten Klooster, Ingrid v Leeuwen,
Nina Scheres, Eloise C Anthony
and Peter L Hordijk*

Sanquin Research and Landsteiner Laboratory, Academic Medical
Center, University of Amsterdam, Plesmanlaan, Amsterdam,
The Netherlands

The Rho GTPase Rac1 controls cell adhesion and motility. The effector loop of Rac1 mediates interactions with downstream effectors, whereas its C-terminus binds the exchange factor β -Pix, which mediates Rac1 targeting and activation. Here, we report that Rac1, through its C-terminus, also binds the nuclear oncogene SET/I2PP2A, an inhibitor of the serine/threonine phosphatase PP2A. We found that SET translocates to the plasma membrane in cells that express active Rac1 as well as in migrating cells. Membrane targeting of SET stimulates cell migration in a Rac1-dependent manner. Conversely, reduction of SET expression inhibits Rac1-induced migration, indicating that efficient Rac1 signalling requires membrane recruitment of SET. The recruitment of the SET oncogene to the plasma membrane represents a new feature of Rac1 signalling. Our results suggest a model in which Rac1-stimulated cell motility requires both effector loop-based downstream signalling and recruitment of a signalling amplifier, that is, SET, through the hyper-variable C-terminus.

The EMBO Journal (2007) **26**, 336–345.

doi:10.1038/sj.emboj.7601518

Subject Categories: signal transduction

Keywords: migration; Rac1; SET

Introduction

Directional cell migration is initiated by polarisation of the cell body, which is regulated by the actin and microtubule cytoskeleton (Ridley *et al*, 2003). Subsequent integrin-mediated adhesion and de-adhesion as well as membrane turnover and vesicle transport further promote efficient motility (Ridley *et al*, 2003). In all these different processes, small GTPases of the Ras, Rab, Arf and Rho families cooperate (Oxford and Theodorescu, 2003; Ridley *et al*, 2003). The Ras-related Rap proteins mediate inside-out signalling towards β_1 and β_2 integrins (Bos, 2005), the Rab and Arf GTPases regulate vesicle transport and microtubule dynamics (Jordens *et al*, 2005; Kahn *et al*, 2005) and the Rho GTPases

are involved in coordinating the dynamics of the actin cytoskeleton (Bishop and Hall, 2000). Although the various members of these protein families share high levels of sequence homology, they clearly have different functions. Therefore, it is important to understand the mechanisms that control the signalling specificity of small GTPases.

Small GTPases are molecular switches that cycle between a GDP-bound 'off' state and a GTP-bound 'on' state (Rossman *et al*, 2005). This cycling is regulated by GTPase-activating GEFS (guanine-nucleotide exchange factors) and GTPase-inactivating GAPs (GTPase-activating proteins). Whereas active Rho-like GTPases are localised at the plasma membrane (del Pozo *et al*, 2000), the GDP-bound forms are bound to RhoGDI (Guanine-nucleotide Dissociation Inhibitor), which is a cytosolic protein (Olofsson, 1999; del Pozo *et al*, 2002).

Once activated by an exchange factor, Rho-like GTPases expose their effector loop, which can then bind a wide range of effector proteins, leading to their activation and downstream signalling (Hall, 1998; Bishop and Hall, 2000). For Rac1, these include PAK (p21-activate kinase), IQ-GAP, POSH, POR1, WASP, p67PHOX and Sra-1 (Kobayashi *et al*, 1998; Bishop and Hall, 2000). However, the effector loop of Rac1 is very similar and sometimes even identical to that of other Rho GTPases that show clearly distinct functions. Thus, the interactions, mediated via the effector loop, do not fully explain the differential signalling by Rho GTPases. Specific, subcellular targeting of Rho GTPases is therefore likely to provide another, important means to control localised interactions with specific effector proteins.

The hypervariable C-terminus of Rho GTPases mediates subcellular localisation (Michaelson *et al*, 2001; Klooster *et al*, 2006) and has been implicated in signalling specificity, presumably owing to its role in GTPase targeting (Prieto-Sanchez and Bustelo, 2003; van Hennik *et al*, 2003; Klooster *et al*, 2006). This C-terminal domain comprises 10–15 amino acids, positioned just upstream of the CAAX-box that mediates the attachment of a lipid anchor. Recently, we showed that Rac1, through its C-terminus, binds directly to the Rac1/Cdc42 GEF β -Pix (Klooster *et al*, 2006). β -Pix recruits Rac1 to membrane ruffles and focal adhesions in the leading edge of polarised, migrating cells. Thus, β -Pix mediates both targeting and localised activation of Rac1. We also found that PAK1 and Rac1 compete for binding to the β -Pix SH3 domain and that activation of PAK1 allows increased β -Pix–Rac1 binding, targeting and Rac1 signalling (Klooster *et al*, 2006).

Rac1-mediated activation of PAK1 triggers a signalling pathway comprising p38 mitogen-activated kinase (p38MAPK) (Zhang *et al*, 1995). p38MAPK can be inactivated by the ubiquitous serine/threonine phosphatase PP2A (Sundaresan and Farndale, 2002), which resides in the nucleus and cytoplasm as well as at the plasma membrane (Lechward *et al*, 2001). In addition, PP2A can associate with and dephosphorylate PAK kinases (Westphal *et al*, 1999; Zhan *et al*, 2003), suggesting that PP2A represents a 'brake' on the Rac1–PAK–p38 MAPK pathway.

*Corresponding author. Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Plesmanlaan, 1066 CX, Amsterdam, The Netherlands.
Tel.: +31 20 512 3263; Fax: +31 20 512 3474;
E-mail: p.hordijk@sanquin.nl

Received: 12 July 2006; accepted: 29 November 2006

Here, we show that Rac1 associates directly to the PP2A inhibitor SET/I2PP2A (von Lindern *et al*, 1992; Li *et al*, 1995), a protein that has also been implicated in histone acetylation (Seo *et al*, 2001) and gene transcription (Miyamoto *et al*, 2003). SET is primarily a nuclear protein that, depending on its state of phosphorylation, can also localise to the cytoplasm. Intriguingly, Rac1 activation recruits phosphorylated SET to the plasma membrane, where SET acts as a signalling amplifier for Rac1-mediated cell migration.

Results

The hypervariable domain of Rac1 interacts with the nucleosome assembly protein domain of SET

Recently, we established the relevance of the hypervariable C-terminus of Rac1 for its subcellular targeting, activation and consequent signalling. Specifically, we identified the Rac1/Cdc42 exchange factor β -Pix as an important Rac1-targeting protein (Klooster *et al*, 2006). In the course of these studies, we identified several other proteins that interact selectively with the Rac1 C-terminus. One of the most abundant of these (Figure 1A) was isolated and identified by N-terminal sequencing. This way, the nuclear oncogene SET was identified as a novel Rac1-binding protein. SET has a calculated mass of 32 kDa, but migrates at a higher apparent molecular weight on SDS-PAGE. This may be due to phosphorylation (see below) or, more likely, to its extremely acidic C-terminus.

The interaction between Rac1 and endogenous SET was confirmed by streptavidin-based pull-down assays using the biotinylated C-terminus of Rac1 (Figure 1B). Parallel assays using the hypervariable C-termini of Rac2 and Cdc42 showed that these do not bind SET, indicating that the interaction with Rac1 is specific. Similarly, we found that an HA-tagged SET protein, expressed in Cos7 cells, interacts selectively with the C-terminus of Rac1, but not with those of Rac2 or Cdc42 (Figure 1C).

Next, we isolated recombinant Rac1 as well as GST-SET from bacterial lysates and performed pull-down assays with these purified proteins. These data show that Rac1 binds to GST-SET, but not to GST (Figure 1D), indicating that also the full-length proteins interact with each other. Furthermore, the use of purified proteins shows that the interaction between Rac1 and SET is direct.

The relevance of the Rac1-SET interaction was further investigated by testing whether endogenous Rac1 binds to endogenous SET. This was tested by isolating Rac1 from cell lysates with either GST-RhoGDI or with a biotinylated peptide derived from the PAK1-CRIB domain (Price *et al*, 2003). RhoGDI is a protein that recognises inactive, GDP-bound Rac1 and normally retains this GTPase in the cytosol, whereas the PAK1-CRIB domain specifically interacts with active, GTP-bound Rac1. From these experiments, it is clear that SET can be found in complex with GST-RhoGDI as well as with PAK1-CRIB, but not with GST or the control peptide (Figure 1E). This indicates that an endogenous Rac1-SET complex can form in cells, and that the interaction appears to be nucleotide-independent. Of note, the C-terminus of Rac1 is, even when Rac1 is bound to RhoGDI, exposed (Grizot *et al*, 2001) and capable of forming complexes with other proteins, as we found previously for β -PIX (Klooster *et al*, 2006). The presence of multiple proteins detected by the SET

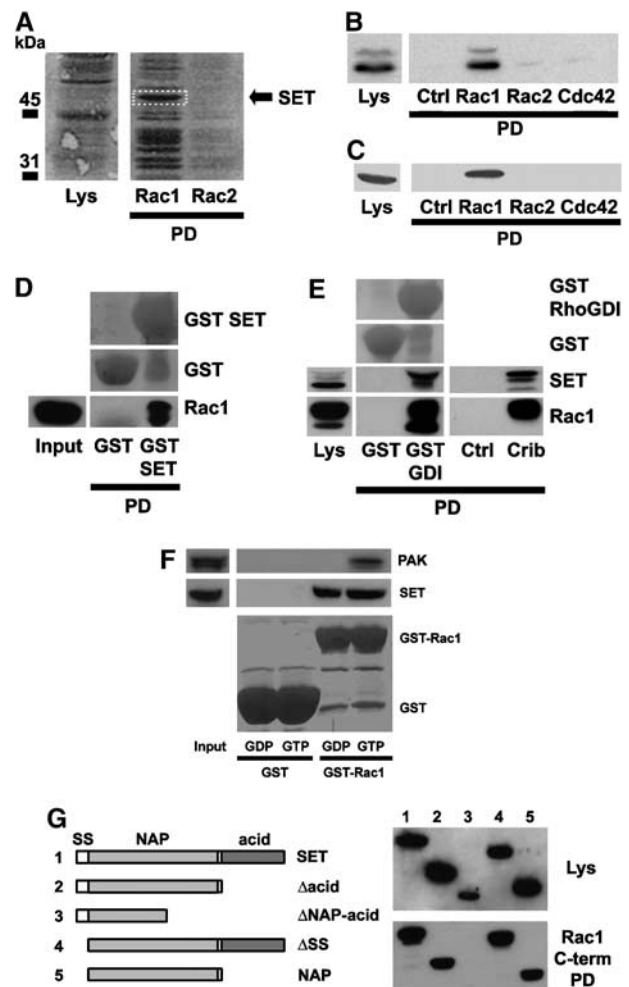


Figure 1 Rac1 interacts with the nuclear oncogene SET. (A) Identification of the Rac1-interacting protein SET. Streptavidin-based pull-down assays (PD) were performed with biotinylated peptides encoding the hypervariable domain of Rac1 and Rac2. Total cell lysates (Lys) and Rac-interacting proteins were detected by Western blotting followed by Coomassie staining. The protein band indicated by the dotted square was identified by peptide sequencing as SET. (B, C) SET interacts specifically with the C-terminus of Rac1. Streptavidin-based pull-down assays were performed with a control peptide (Ctrl) or the C-terminal peptides derived from Rac1, Rac2 and Cdc42 in lysates from HeLa cells (B) or from Cos7 cells, expressing HA-tagged SET (C). Samples were blotted and immunostained for endogenous (B) or HA-tagged (C) SET. Lys, cell lysates blotted for SET (B) or HA (C). (D) Purified, full-length Rac1 interacts directly with full-length SET. Rac1, GST and GST SET were purified from bacteria. Pull-down assays were performed with GST and GST-SET in the presence of Rac1. Isolated proteins were subsequently blotted and immunostained for Rac1 and GST. Input represents the Rac1 protein, added to the assay. (E) Endogenous Rac1 binds to endogenous SET. Rac1 was isolated from HeLa cells with GST RhoGDI or the Crib domain of PAK1 and subsequently immunostained for endogenous SET and Rac1. Controls represent GST alone or a control peptide (Ctrl). (F) The SET-Rac1 interaction is nucleotide independent. Cos7 lysates expressing HA-tagged SET were incubated with GST or GST-Rac1 loaded with either GDP or GTP γ S. Isolated proteins were subsequently blotted and immunostained for PAK, HA and GST. Input represents SET or PAK protein, added to the assay. Detection of two bands for PAK is due to PAK isoforms. (G) The C-terminus of Rac1 interacts with the NAP domain of SET. Streptavidin-based pull-down assays were performed with the C-terminal Rac1 peptide in Cos7-derived cell lysates expressing different MYC-tagged SET mutants, indicated in the left panel, and subsequently immunostained with anti-MYC.

antibody on Western blot is likely due to expression of SET isoforms (Li *et al*, 1996; Santa-Coloma, 2003).

To further confirm that the Rac1–SET interaction is independent of the nucleotide bound to Rac1, we incubated *Escherichia coli*-derived GST-Rac1, loaded with either 10 μ M GDP or 10 μ M GTP γ S, with Cos7-derived lysates expressing HA-tagged SET, followed by a GST pull-down assay. These results of the experiment also show that Rac1 associates with SET in a nucleotide-independent fashion (Figure 1F), similar to our earlier findings on the interaction between Rac1 and β -Pix (Klooster *et al*, 2006). In contrast, binding to Rac effector PAK was observed only with the GTP-loaded Rac1 protein (Figure 1F).

The SET protein comprises a nucleosome assembly protein (NAP) domain, a C-terminal acidic domain (Acid) and an N-terminal region, which comprises serine phosphorylation sites (SS) (Figure 1G). In order to test which of these domains is involved in the Rac1–SET interaction, we generated several deletion mutants (Figure 1G, mutants 1–5) and expressed them in Cos7 cells. Subsequently, we tested whether the biotinylated Rac1 C-terminus could associate to any of these SET mutants. The results show that SET-mutant 3 lacking the acid domain and part of the NAP domain (Δ NAP-acid) can no longer interact with the hypervariable domain of Rac1 (Figure 1G, mutant 3). As neither the acid domain nor the SS domain is required for Rac1 binding (Figure 1G, mutants 4, 5) these data indicate that the NAP domain of SET mediates the interaction with the C-terminus of Rac1.

SET translocates to the plasma membrane upon Rac1 activation

SET is primarily a nuclear protein. As Rac1 is mainly cytosolic and, when activated, localises at the plasma membrane, we tested whether the localisation of SET would be altered in the presence of Rac1. We coexpressed L61Rac1-RFP (an activated Rac1 mutant) and MYC-tagged SET in HeLa and HEK293 cells. In line with published data, we found that SET localised primarily to the nucleus (Figure 2A, left cell). However, in cells where SET was coexpressed with active Rac1, we observed that a significant fraction of the total pool of the SET protein was no longer present in the nucleus but could be found at the plasma membrane, colocalising with Rac1 (Figure 2A, cell on the right).

To further explore the finding that Rac1 activity directs SET to the plasma membrane, we coexpressed SET with β -Pix, an activator of Rac1, or with the inactive N17Rac. Interestingly, expression of β -Pix (Figure 2B) also induced the translocation of SET to the plasma membrane, whereas expression of N17Rac1 (Figure 2C) did not. This further indicates that Rac1 activation is required to induce membrane recruitment of SET. In addition, β -Pix is also an exchange factor for Cdc42. However, activated Cdc42 could not induce membrane targeting of SET (data not shown), which shows that SET translocation is specific for Rac1. This is in good agreement with our finding that SET does not bind to the hypervariable domain of Cdc42 (Figure 1B and C). Moreover, β -Pix activates Rac1 at the plasma membrane and not in the nucleus, suggesting that Rac1 activity at the plasma membrane is required to induce translocation of SET. To explore this further, we expressed a constitutively active Rac1 mutant with a mutation in the CAAX box (V12Rac1C189S). This non-

geranylgeranylated mutant localises mainly to the nucleus (Klooster *et al*, 2006). Coexpression with SET did not induce translocation of SET to the plasma membrane (not shown). This also confirms that Rac1 needs to be activated and localised at the plasma membrane to induce SET translocation. Finally, the localisation of SET in membrane ruffles, as seen in migrating cells or in cells expressing active Rac1 or Pix, was not transient; SET was found in membrane ruffles, irrespective of the time point of the analysis.

Currently available antibodies precluded detection of the Rac1-induced translocation of endogenous SET. However, the SET-related protein pp32 forms a stable complex with SET (Seo *et al*, 2001) and thus could translocate, together with SET, to the plasma membrane. Indeed, expression of L61Rac1 in HeLa cells showed that endogenous pp32, like SET, also translocates to the plasma membrane upon activation of Rac1 (not shown).

Rac1 activity is increased in migrating cells. We therefore asked whether the localisation of SET is different in migrating versus stationary cells. This was tested by expressing SET in HeLa cells that were subsequently induced to migrate in a ‘scratch’ assay. Whereas SET remained nuclear in stationary cells, localised in the monolayer, we found pronounced staining of SET at the plasma membrane, already after 1 h following wounding, in cells that showed formation of lamella and were migrating into the wound (Figure 3). This suggests that activation of endogenous Rac1 also induces the translocation of SET to the plasma membrane. To further confirm this, we reduced endogenous Rac1 expression by transient transfection of siRNA, before performing a migration/scratch assay on SET-cotransfected cells. Figure 3B shows that under these conditions, SET no longer translocates to the plasma membrane in cells that are at the edge of a wound.

Serine phosphorylation of SET regulates Rac1 binding and membrane targeting

SET is subject to serine phosphorylation, in particular on position 9 (Adachi *et al*, 1994). However, the functional relevance for this phosphorylation is unknown. To determine whether Ser-9 phosphorylation has any effect on the localisation of SET or its interaction with Rac1, we used site-directed mutagenesis to mutate Ser-9 to either alanine (A9) or glutamic acid (E9), mimicking respectively the non-phosphorylated or phosphorylated form of SET. GST-fusion proteins of these mutants were purified from bacteria and were subsequently tested for binding to wild-type (WT) Rac1. Western blot analyses showed that Rac1 preferably interacts with the E9 mutant and showed very little binding to the A9 mutant (Figure 4A). This indicates that the interaction between Rac1 and SET is mainly determined by the phosphorylation of SET at serine 9.

In addition to serine phosphorylation, the N-terminus of SET is also involved in dimerisation (Miyaji-Yamaguchi *et al*, 1999). To test a possible correlation between these events, we coexpressed HA-tagged WT SET with MYC-tagged A9 or E9 SET mutants and performed anti-MYC immunoprecipitations (IPs). The results show that only the A9 mutant interacts with WT SET (Figure 4B), which suggests that phosphorylation of Ser-9 prevents dimerisation. Similar results were obtained using GST-SET A9/E9 mutants (not shown).

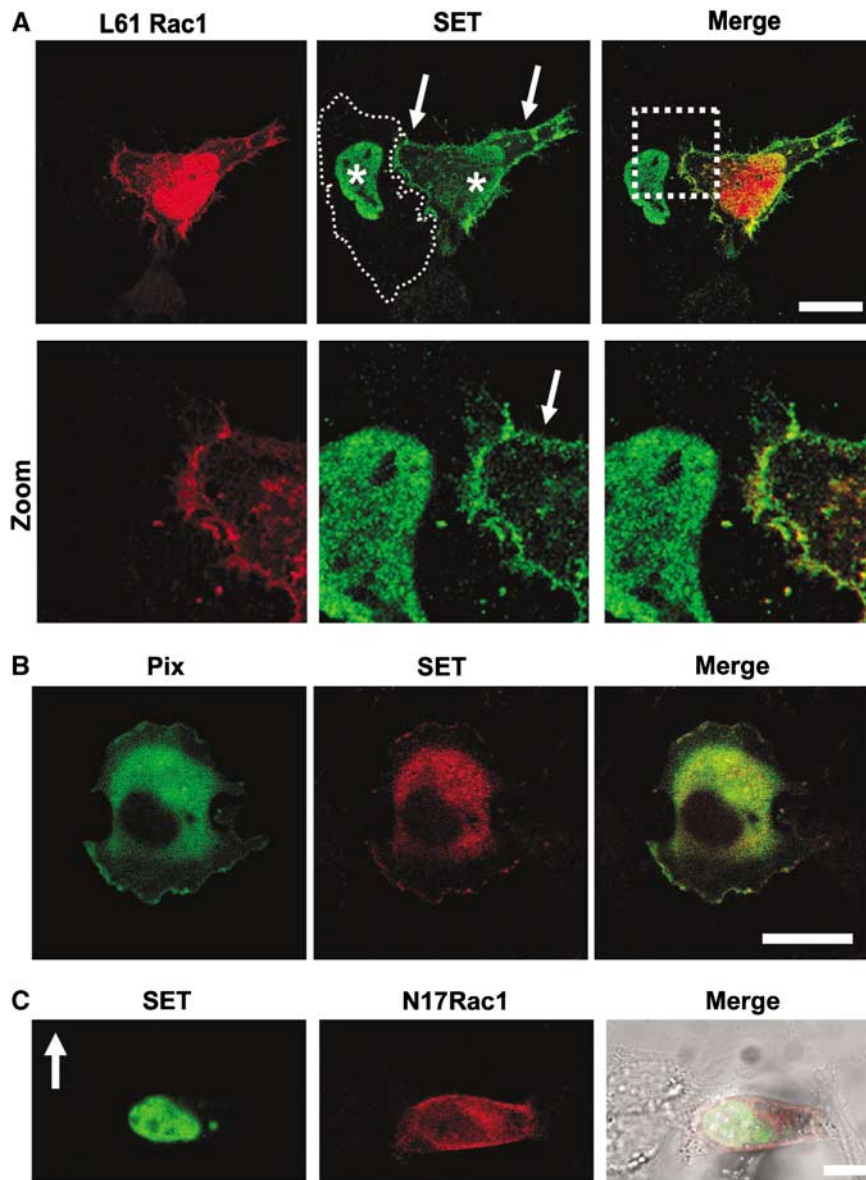


Figure 2 Active Rac1 induces translocation of SET from the nucleus to the plasma membrane. (A) HeLa cells expressing constitutively active Rac1 (red) and SET (green). Note the difference in the localisation of SET in the single SET-transfected cell (left) and the double transfected cell (right, cell body indicated by the dashed line). Dashed box in the merged image indicates the enlarged area, which is shown in the lower panels. Asterisks indicate nuclei; arrow indicates SET localisation at the plasma membrane. (B) HeLa cells expressing β -Pix (red) and SET (green). (C) HeLa cells expressing dominant-negative N17Rac1 (red) and SET (green). The merge also contains the corresponding phase-contrast image to show the outline of the cell. Scale bars, 10 μ m.

SET is a specific inhibitor of the phosphatase PP2A (Li *et al*, 1996). As Ser-9 phosphorylation affects dimerisation as well as Rac1 binding, we also tested whether GST-SET A9/E9 mutants show differential binding to endogenous PP2A. The results show that PP2A only binds the GST-SET E9 mutant (Figure 4C). In line with these data, PP2A could also be clearly detected in association with the Rac1 C-terminus (Figure 4D). The latter finding most likely represents the formation of a trimeric Rac1–SET–PP2A complex, as Rac1 and SET interact directly (Figure 1).

L61Rac1 induces the translocation of SET from the nucleus to the plasma membrane (Figure 2). As Rac1 preferentially binds the SET E9 mutant, we compared the subcellular distribution of the SET A9/E9 mutants and the *WT protein in the absence or presence of activated Rac1 (Figure 4E and

F). These experiments showed that the A9-SET was largely resistant to Rac1-induced membrane translocation, whereas the E9 and WT proteins were recruited by activated Rac1 and showed similar subcellular distributions. Moreover, these experiments showed that the SET E9 mutant already localises in the nucleus as well as in the cytosol. This shows that phosphorylation of SET at Ser-9 and hence its monomerisation is sufficient to induce cytosolic localisation of a fraction of cellular SET, but is not sufficient to induce membrane targeting of SET (Figure 4E), the latter event being dependent on Rac1 activity (Figure 4E). This also suggests that phosphorylation and monomerisation of SET is a step that does not require active Rac1. Indeed, we observed that expression of V12Rac1 does not influence SET dimerisation (data not shown) and therefore does not appear to regulate SET

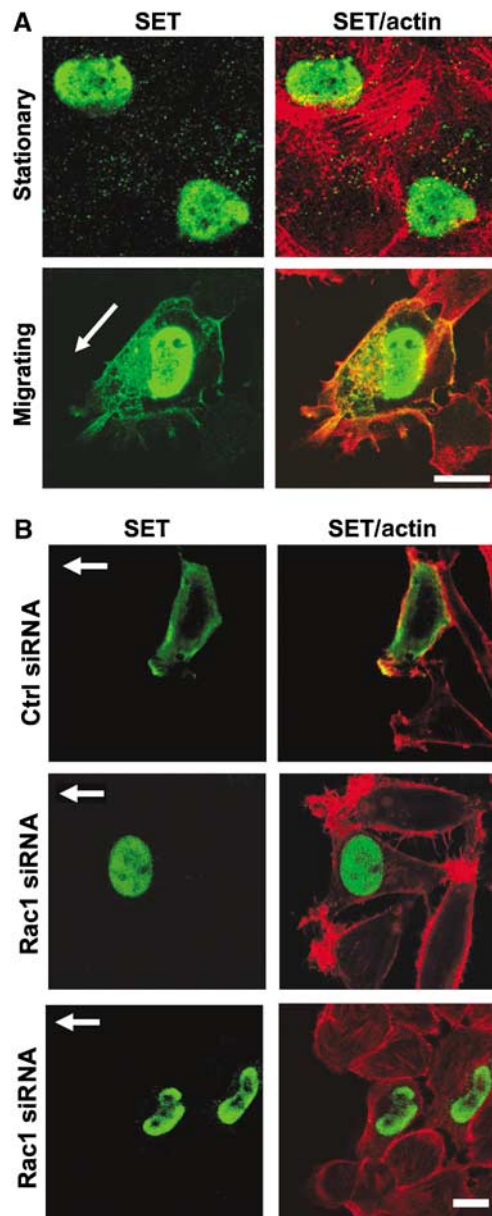


Figure 3 SET translocates to the plasma membrane in migrating cells. (A) SET-transfected HeLa cells, either growing in a monolayer (stationary, upper panels) or induced to migrate into a wound (migrating, lower panels), were immunostained for SET (green) and counterstained for F-actin (red). The direction of migration is indicated by the arrow. Scale bar, 10 μ m. (B) HeLa cells were cotransfected with SET and the pSUPER vector with either control siRNA (Ctrl) or Rac1 siRNA, of which two examples are shown. SET is indicated in green and actin in red. Arrows indicate the direction of migration. Scale bar, 10 μ m.

phosphorylation. This suggests that Rac1-mediated membrane targeting of SET is not regulated via increased phosphorylation of SET, but rather by Rac1 forming a docking site for SET at the plasma membrane.

Together, this data suggest that unphosphorylated SET can form a dimer that dissociates upon phosphorylation of Ser-9. This mutant partially translocates to the cytosol, allowing complex formation with PP2A and Rac1, resulting in further recruitment to the plasma membrane upon cell stimulation.

SET and Rac1 cooperate in migration

The recruitment to the plasma membrane of a primarily nuclear protein by activated Rac1 represents a novel feature of Rho GTPase signalling. In order to determine the biological significance of this phenomenon, we tested whether membrane association of the SET protein modulates Rac1 signalling. We therefore constructed a SET protein fused to an N-terminal myristoylation sequence (MYR-SET). Expression of MYR-SET in HEK293 cells showed that the protein, in sharp contrast to WT-SET, primarily localised to the plasma membrane and did not show nuclear localisation (Figure 5A and B). Of note, expression of MYR-SET did not affect the overall morphology of the cells. Moreover, we could not detect a difference in the association of WT-SET or MYR-SET to the Rac1 C-terminus (not shown).

To test the effect of membrane-associated SET on cell migration, we seeded HeLa cells expressing either empty vector, WT-SET or MYR-SET on fibronectin-coated 5 μ m pore size polycarbonate Transwell filters and allowed them to migrate towards a gradient of 10% fetal calf serum (FCS). These experiments showed that there is no significant effect of WT-SET on the migration of HeLa cells when compared to the empty retroviral vector (EV) cells (Figure 5C). However, the membrane-targeted MYR-SET protein induced a four-fold increase in the migration of HeLa cells towards FCS (Figure 5C). Interestingly, MYR-SET-induced migration was still blocked by the Rac1 C-terminus (Figure 5C), suggesting that membrane-targeted SET requires localised Rac1 signalling to induce migration.

Rac1 mediates cell adhesion, spreading and migration (Anand-Apte *et al*, 1997; Price *et al*, 1998). To test the role of SET in these processes, we generated HeLa cells with reduced expression of SET by means of siRNA. Adhesion on fibronectin-coated gold electrodes was quantified as described (Klooster *et al*, 2006) using ECIS. The data show that reduced expression of SET does not impair the adhesion and spreading of these cells (Figure 5D). In addition, we performed similar experiments with cells expressing WT-SET or MYR-SET on either fibronectin or collagen. We found that neither WT-SET nor MYR-SET altered adhesion and spreading of either HeLa or HEK293 cells (not shown). This suggests that SET does not play a role in cell adhesion and/or spreading.

To further establish the role of SET in Rac1-mediated migration, we expressed V12Rac1 in control cells and in the SET-knockdown cells. Expression of activated Rac1 enhanced migration towards FCS approximately eight-fold in control cells when compared to EV cells. Interestingly, Rac1 induced migration in cells where expression of SET (V12Rac1/siSET) was significantly reduced, compared to the EV cells (Figure 5E) and control siRNA (not shown). Basal migration of the siRNA against SET (siSET) cells was comparable to migration of the EV cells (Figure 5E). This indicates that Rac1-induced migration is dependent on SET.

In conclusion, the above findings show that Rac1 activation recruits the PP2A inhibitor SET to the plasma membrane. The Rac1-SET complex subsequently cooperates to induce cell motility. These data demonstrate that SET recruitment serves to amplify Rac1 signalling at the plasma membrane.

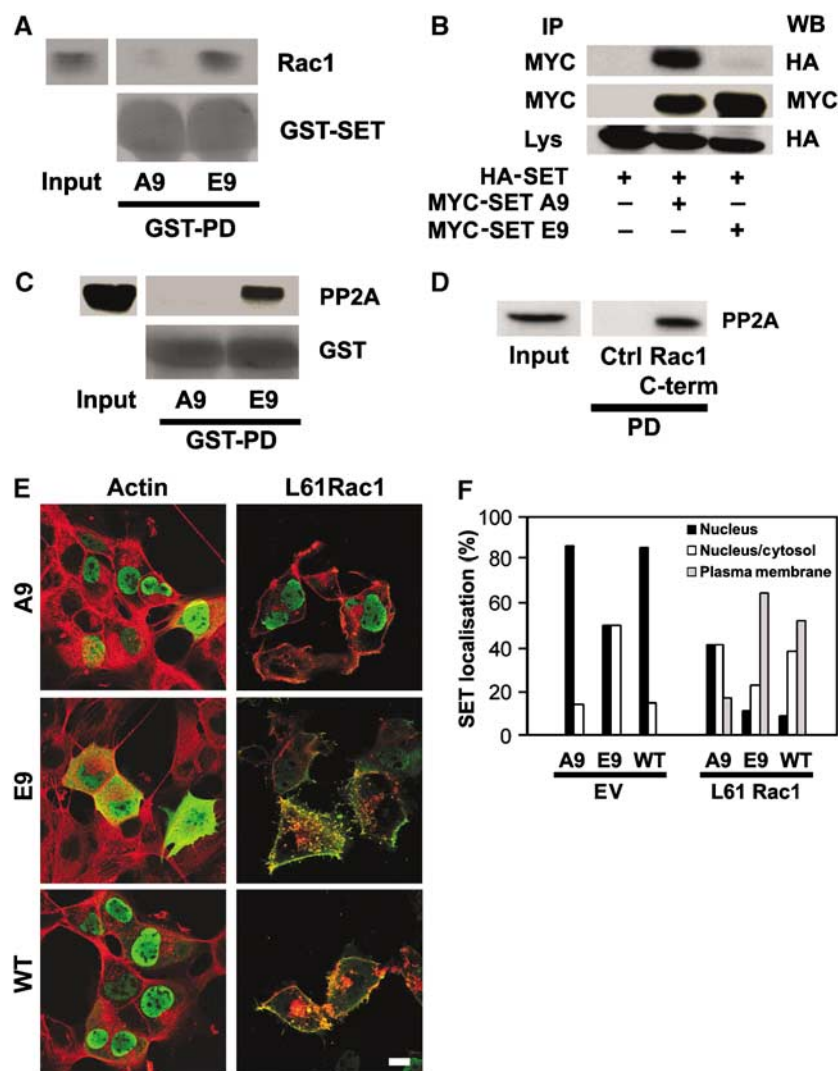


Figure 4 Serine phosphorylation of SET regulates its interaction with Rac1 and PP2A. **(A)** The phospho-mimetic SET E9 mutant binds Rac1. GST-SET A9 and GST-SET E9 were purified from bacteria and subsequently incubated with Cos7 cell lysates expressing Rac1 (Input). GST pull-downs were analysed by Western blotting for Rac1. **(B)** The phospho-mimetic SET E9 mutant does not dimerise. HA-tagged WT-SET was coexpressed with MYC-tagged SET A9 and E9 in Cos7 cells. Immunoprecipitations (IPs) with anti-MYC were performed and lysates (Lys) and IPs were immunostained with both anti-MYC and anti-HA. **(C)** The SET E9 mutant interacts with PP2A. GST-SET A9 and -E9 mutant proteins were purified from bacteria and incubated with Cos7-derived cell lysates (input). Endogenous PP2A was detected following GST pull-down assay and Western blotting by anti-PP2A staining. **(D)** The hypervariable domain of Rac1 associates with PP2A. Streptavidin-based pull-down assays with a biotinylated control peptide (Ctrl) or the C-terminal domain of Rac1 were performed in HeLa cell lysates (input). Endogenous PP2A was detected following Western blotting of the isolated proteins by anti-PP2A. **(E)** Phospho-deficient SET A9 mutant does not translocate in the presence of activated Rac1. Merged images show SET A9, E9 and WT SET (green) in HeLa cells, coexpressed with empty vector (left panels) or activated L61Rac1 (in red, right panels). F-actin is shown in red (left panels only). Scale bar, 10 μ m. **(F)** Quantification of SET localisation was determined by immunostaining in combination with confocal analysis (E) and divided in three categories: nuclear staining (black bars), nuclear and cytosol (white bars) and plasma membrane (grey bars). Quantification of the SET distribution is based on counting at least 50 cells per group.

Discussion

This study provides evidence for a new and unexpected role for the oncogene SET/I2PP2A in Rac1-driven cell migration. Despite its small size and the lack of well-established signalling domains, SET is a versatile regulator of various (patho)-physiological processes including histone acetylation (Seo *et al*, 2001), regulation of transcription (Seo *et al*, 2001), cell growth (Canela *et al*, 2003) and transformation (Kumar *et al*, 2004). In addition, SET is an inhibitor of the tumour suppressors Nm23-H1 (Fan *et al*, 2003), a granzyme A-activated DNase, and the serine/threonine phosphatase

PP2A (Li *et al*, 1996). Finally, SET/I2PP2A is upregulated in Alzheimer's disease (Tanimukai *et al*, 2005), mediates neuronal apoptosis (Madeira *et al*, 2005) and has been found as a SET-CAN fusion in acute myeloid leukaemia (von Lindern *et al*, 1992). Our data add a novel function to this list, that is, the amplification of Rac1-induced signalling towards cell motility.

The Rac1-induced recruitment of SET to the plasma membrane represents an intriguing result from the current study. This translocation appears to be dependent on SET phosphorylation and monomerisation, as this leads to a partial exit from the nucleus (Figure 4). Phosphorylated SET could

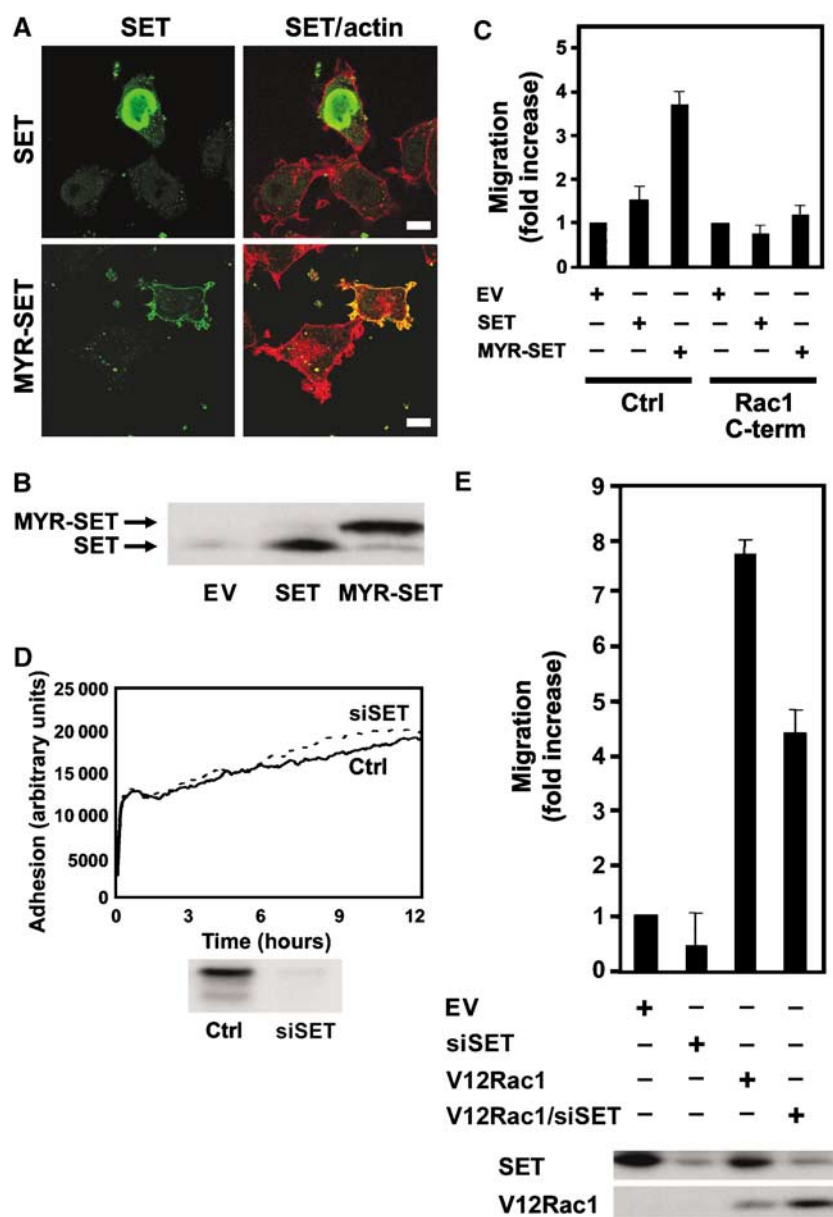


Figure 5 Rac1 and SET cooperate in cell migration. (A) Images show transfected HEK293 cells immunostained for SET or MYR-SET (green) and counterstained for F-actin (red). Scale bars, 10 μ m. (B) Western blot analysis of HEK293-derived cell lysates expressing SET or MYR-SET. (C) Membrane-targeted SET enhances migration. HeLa cells expressing either the empty retroviral vector (EV), SET or MYR-SET were allowed to migrate across a fibronectin-coated Transwell filter towards 10% FCS for 4 h. During migration, cells were incubated with a cell-permeable control peptide (Ctrl) or the C-terminal peptide of Rac1 (0.2 mg/ml). Migration is indicated as fold increase relative to the migration of the empty vector-expressing cells. Western blot analysis shows expression of SET and MYR-SET in HeLa cells. (D) Reduction of SET expression does not impair cell adhesion/spreading. HeLa cells, transduced with control siRNA (Ctrl) or siSET, were seeded on fibronectin-coated gold electrodes and analysed by ECIS for 12 h. Western blot analysis shows expression of endogenous SET in retrovirally transduced HeLa cells expressing control siRNA (Ctrl) or siSET. (E) Rac1-induced migration is SET-dependent. HeLa cells expressing either the empty retroviral vector (EV), siSET, V12Rac1 or V12Rac1 in combination with the SET siRNA were allowed to migrate in a Transwell system towards 10% FCS for 4 h. Migration is indicated as fold increase relative to the migration of the empty vector-expressing cells. Western blot analysis shows expression of endogenous SET and V12Rac1 in HeLa cells without or with expression of the siRNA.

associate with Rac1 already in the cytosol, that is, when Rac1 is still inactive and bound to RhoGDI (Figure 1E). Rac1 activation further stimulates recruitment of phosphorylated SET to the plasma membrane.

Whereas the hypervariable C-terminus of Rho-like GTPases has been implicated in subcellular targeting, our results on the Rac1-SET interaction suggest an additional function for this domain. Knockdown of SET expression, which was approximately 70–80% efficient (Figure 5E), signi-

ficantly blocked cell migration induced by activated Rac1 (Figure 5E). This suggests that SET acts as a downstream effector of Rac1. However, whereas constitutive membrane association of SET induces cell migration, this response was still dependent on the Rac1-SET interaction, suggesting that SET targeting to the membrane is not sufficient to bypass Rac1 signalling. Our findings therefore indicate that SET recruitment to the membrane and its binding to the Rac1 C-terminus serve to locally amplify Rac1 signalling. SET-

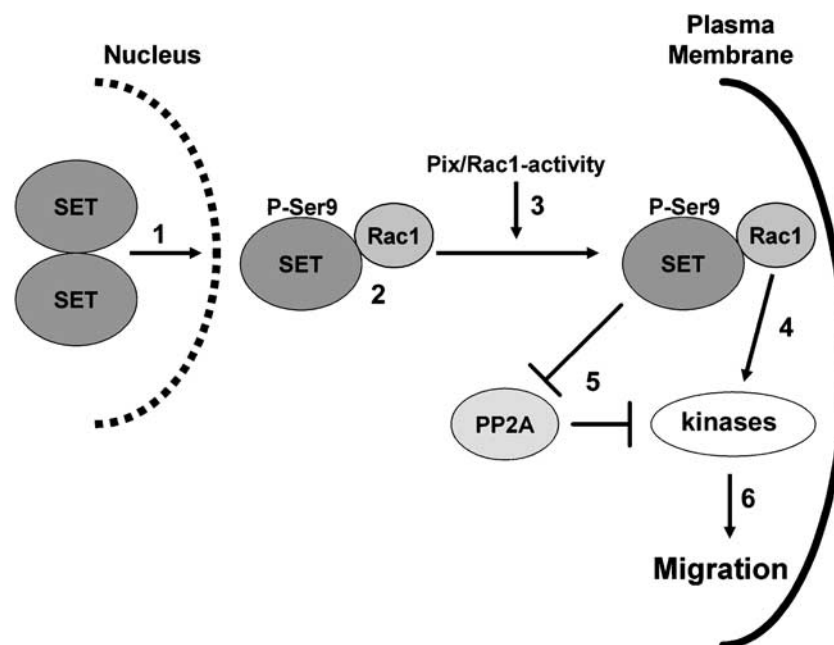


Figure 6 Model for SET acting as a signalling amplifier for Rac1. In its unphosphorylated form, SET is primarily nuclear and forms a dimer (1). Phosphorylation of SET at serine 9 dissociates the dimer and allows SET redistribution to the nucleus as well as the cytoplasm (2). This may allow interaction with Rac1 in the cytoplasm. Upon activation of Rac1, for example, by the Rac-GEF β -Pix, SET translocates to the plasma membrane (3) where active Rac1 can stimulate kinase-mediated signalling (4). This requires SET-mediated inhibition of the serine/threonine phosphatase PP2A (5). Kinase-driven signalling is subsequently required for efficient cell migration, which is initiated by Rac1, in a SET-dependent fashion (6).

mediated inhibition of PP2A, which negatively regulates Rac1 signalling, is a likely mechanism. However, SET is a multi-functional protein and we cannot exclude other mechanisms or protein-protein interactions by which SET stimulates Rac1 action (Figure 6).

There is abundant nucleo-cytoplasmic trafficking of proteins. In response to receptor-mediated signalling, proteins such as the MAPKs (Kytosseva, 2004) or the STATs (Heim, 1999) translocate from the membrane or the cytoplasm to the nucleus. In contrast, there are very few examples of, primarily nuclear, proteins that, in response to cell stimulation, translocate to the membrane. Wang *et al* (2006) recently showed that activation of LFA1 in T lymphocytes promotes rapid nuclear-to-cytoplasmic translocation of the mRNA-stabilising protein HuR. In addition, the SET-related protein pp32 associates with and regulates PP2A at cellular membranes in neurons and is involved in synaptogenesis (Costanzo *et al*, 2006). In line with these data, we found that pp32, like SET, also translocates to the plasma membrane following Rac1 activation. These findings provide increasing evidence that a group of small, nuclear proteins implicated in transcriptional regulation (Seo *et al*, 2001), mRNA stability/transport (Brennan *et al*, 2000; Wang *et al*, 2006) and inhibition of PP2A (Li *et al*, 1996) play important roles in GTPase signalling at the plasma membrane.

In conclusion, our data suggest a revision of the current model for GTPase action. Similar to the other Rho GTPases, the effector domain of Rac1 mediates interactions with various downstream signal transducers, including PAK kinases, POSH and p67^{phox} (Bishop and Hall, 2000). The hypervariable C-terminal domain appears to act in a similar fashion, specifically binding to a different set of proteins, including

PIP-5-kinase, Crk, β -Pix (Tolias *et al*, 1995; van Hennik *et al*, 2003; Klooster *et al*, 2006) and SET. Some of these (e.g. β -Pix) act as Rac1-targeting and -activating proteins, whereas others (e.g. SET) promote efficient downstream signalling. Thus, by combining the functions/interactions mediated by the effector loop with those mediated by the hypervariable C-terminus, Rho GTPases control efficient, localised signal transduction.

Materials and methods

Antibodies and constructs

Antibodies: Anti-HA-tag (12CA5) was from Boehringer Mannheim Corp. Anti-MYC-tag (sc-789) and anti-SET (sc-5655) were obtained from Santa Cruz. Anti-Rac1 (610651) was obtained from Transduction Laboratories. Anti-PAK (2604) was from Cell Signalling. F-actin was stained with rhodamine-labelled phalloidin (Molecular Probes). **Plasmids:** GFP- β -Pix WT and L61Rac1-RFP have been described previously (Klooster *et al*, 2006), V12Rac1-HA and N17Rac1 were obtained from Guthrie (Guthrie Healthcare System, Sayre, PA). GST-Rac1 was a kind gift from Dr Ahmadian (Haeusler *et al*, 2003) and pRetrosuper (Berns *et al*, 2004) and HA-tagged SET B were a kind gift from Dr R Bernards. SET WT and mutants were generated by PCR and cloned in a MYC-tagged pCDNA3.1 vector and in GST-6P-1 (27-4597-01, Amersham Biosciences Corp., Piscataway, NJ) using the *Bam*HI and *Eco*RI restriction sites. The following primers were used to generate the different SET mutants:

Primer 1 5'TTGGATCCTCGGCGCAGGCGGCCAAAG3'
Primer 2 5'TTGGATCCCAAGAAGCGATTGAACACATTG3'
Primer 3 5'TTGAATTCATTAAGCTTATCCTTTCCAGATTTCATTG3/
Primer 4 5'TTGAATTCATTAAGCTTGTAGTACTGTAATGGGTTTGGC3/
Primer 5 5'TTGAATTCATTAAGCTTGTGCATCTTCTCTTCATCTCC3'

Full-length SET B was amplified with primers 1 and 5, Δ acid with 1 and 4, Δ NAP-acid with 1 and 3, Δ SS with 2 and 5 and the NAP domain with 2 and 4. For generating the SET mutant with the

myristoylation sequence of adenylate-kinase 1 β (MYR SET), we annealed the primers

5'GATCGGAGCAGAAGCTGATCAGCGAGGAGGA3' and 5'TCGAGGAATTCGGATCCCAGGTCCTCTCGC3' and cloned them at the 5'-end of SET using *Bam*HI and *Xho*I digestion.

For knock-down studies of SET, we cloned the following primers in the pRetroSuper:

5'GATCTGGATGAAGGTGAAGAAGATTCTCAAGAGAATCTTCTTCACTTCATCCTTTTA3'
5'AGCTTAAAAAGGATGAAGGTGAAGAAGATTCTTGAATCTTCTTCACCTTCATCA3'

As control we used nonfunctional SET-derived sequences:

5'GATCTTGAGAGTGGTGATCCATCTTTCAAGAGAAGATGGATCACTACTCATTTTA3'
5'AGCTTAAAAATGAGAGTGGTGATCCATCTTCTTGAAGAGATGATCACCCTCTCA3'.

The siRNA constructs for Rac1 were in pSUPER and were a kind gift from John G Collard and Amra Hajdo-Milašinović (The Netherlands Cancer Institute) and will be published elsewhere.

Peptide synthesis

Peptides were synthesised on a Syro II, using Fmoc solid phase chemistry. Peptides encoded a biotinylated protein transduction domain (Biotin-YARAAARQARAG) (Ho *et al*, 2001) followed by the 10 amino acids preceding the CAAX motif of Rac1, Rac2 and Cdc42.

Cell culture

The HEK293, HeLa and COS7 cell lines were maintained in Iscove's modified Dulbecco's medium (IMDM; BioWhittaker) containing 10% heat-inactivated FCS (Gibco) at 37°C and 5% CO₂. Cells were passaged by trypsinisation. HEK293, HeLa and COS7 cells were transiently transfected with FuGENE (Roche); 2 μ g DNA was mixed with 6 μ l FuGENE in 100 μ l IMDM, which was incubated for 15 min at room temperature. Subsequently, 2 ml of IMDM with FCS containing 500 000 cells was added to the DNA-FuGENE mixture, which was then incubated in a six-well plate for 6 h. HEK293 and HeLa cells with stable expression of SET, MYR-SET, V12Rac1 in LZRS and pRetroSuper-siSET were generated by retroviral transduction and subsequent selection with 0.2 mg/ml Zeocine (Invitrogen) for the LZRS-based constructs and 0.5 mg/ml puromycin (Invitrogen) for the pRetroSuper-based constructs. Transfection and production of amphotropic retroviruses and pRetroSuper are described elsewhere (Michiels *et al*, 1995; Berns *et al*, 2004).

Pull-down assays

To assay binding of SET, 10×10^6 HEK293, Cos7 or HeLa cells were seeded 1 day before the experiment. Cells were lysed in lysis buffer A (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 10% glycerol and 1% NP-40) and centrifuged for 10 min at 14 000 r.p.m. and 4°C. The supernatant was incubated with the indicated C-terminal peptides (5 μ g) in the presence of streptavidin-coated beads (Sigma) at 4°C for 1 h while rotating. Beads were washed five times in lysis buffer A and resuspended in 25 μ l SDS sample buffer. SET association was determined by Western blot analysis.

To determine Rac activity, HEK293 or HeLa cells (5×10^6 cells) expressing either EV, SET or MYR-SET were seeded on fibronectin-coated dishes for 1 or 24 h. GTP-bound Rac1 was isolated with biotinylated PAK1-Crib peptide (Price *et al*, 2003). Rac1 binding was detected by Western blot.

References

- Adachi Y, Pavlakis GN, Copeland TD (1994) Identification of *in vivo* phosphorylation sites of SET, a nuclear phosphoprotein encoded by the translocation breakpoint in acute undifferentiated leukemia. *FEBS Lett* **340**: 231–235
- Anand-Apte B, Zetter BR, Viswanathan A, Qiu RG, Chen J, Ruggieri R, Symons M (1997) Platelet-derived growth factor and fibronectin-stimulated migration are differentially regulated by the Rac and extracellular signal-regulated kinase pathways. *J Biol Chem* **272**: 30688–30692
- Berns K, Hijmans EM, Mullenders J, Brummelkamp TR, Velds A, Heimerikx M, Kerkhoven RM, Madiredjo M, Nijkamp W, Weigelt B, Agami R, Ge W, Cavet G, Linsley PS, Beijersbergen RL, Bernards R (2004) A large-scale RNAi screen in human

GST-fusion proteins were purified from BL21 bacteria. Following overnight culture, protein expression was induced with IPTG (0.1 mM, 4 h, 37°C). Bacteria were centrifuged and resuspended in PBS, 1% Triton X-100 and 10% glycerol and lysed by sonication (2 \times 30 s, duty cycle 50% and output 6, Branson Sonifier 250). Lysates were cleared by centrifugation for 15 min at 14 000 r.p.m. and 4°C. GST-fusion proteins were isolated by GSH-coated beads while rotating head-over-head at 4°C for 30 min. Samples were then washed five times with lysis buffer A and used as indicated (25 μ g GST-fusion protein per pull-down). Where indicated, GST-Rac1 was loaded with 10 μ M GDP or GTP γ S in lysis buffer A for 30 min to ensure specific nucleotide loading. When appropriate, the GST tag was cleaved from GST-Rac1 and GST-SET when indicated with PreScission Protease (27-0843-01, Amersham Biosciences).

Confocal and phase-contrast microscopy

Forty-eight hours after transfection, cells were fixed with 3.7% formaldehyde (MERCK) in PBS for 5 min and permeabilised with 0.5% Triton X-100 in PBS. Immunostainings were performed at 37°C for 1 h with the indicated antibodies. Fluorescent imaging was carried out with a Zeiss LSM 510 confocal laser scanning microscope. For phase-contrast microscopy, cells were seeded on fibronectin- or collagen-coated dishes and fixed at the indicated time points. Images were obtained with a Leica DMIL.

Electrical resistance measurements

For ECIS-based adhesion experiments, gold ECIS electrodes were coated with either fibronectin or collagen in 0.9% NaCl for 1 h at 37°C. Next, HeLa cells were seeded at a concentration of 200 000 cells per well in 400 μ l of IMDM with 10% FCS. Electrical cell-substrate impedance was subsequently monitored for up to 12 h with the ECIS equipment (Applied Biophysics, Troy, NY).

Cell migration

Transmigration assays were performed in 6.5 mm, 5- μ m pore Transwell plates (Corning Costar, Cambridge MA). The HeLa cells were pretreated with 200 μ g/ml of the indicated cell-permeable peptides for 5 min and during the migration assay. To start the assay, we added 100 000 cells in IMDM to the upper compartment of the Transwell and allowed the cells to migrate towards IMDM with 10% FCS, present in the lower compartment for 4 h. Next, the cells in the upper compartment were removed with a cotton swab and the cells in the lower compartment were fixed and stained with Sytox Orange (Molecular Probes). The migrated cells were counted manually after confocal analysis.

Acknowledgements

We thank Rene Bernards (Netherlands Cancer Institute, Amsterdam) and Reza Ahmadian (Max Planck Institute, Dortmund) and John Collard and Amra Hajdo-MilasinoVIC (Netherlands Cancer Institute, Amsterdam) for providing us with the various cDNAs. In addition, we thank Marian Kroos and Arnold Reuser (Erasmus University, Rotterdam) for peptide sequencing of SET. JPTK is supported by grant no. 203 of the Landsteiner Foundation of Bloodtransfusion Research. PLH and ECA are supported by the Landsteiner Foundation of Bloodtransfusion Research (grant no. 112).

cells identifies new components of the p53 pathway. *Nature* **428**: 431–437

Bishop AL, Hall A (2000) Rho GTPases and their effector proteins. *Biochem J* **348** (Part 2): 241–255

Bos JL (2005) Linking Rap to cell adhesion. *Curr Opin Cell Biol* **17**: 123–128

Brennan CM, Gallouzi IE, Steitz JA (2000) Protein ligands to HuR modulate its interaction with target mRNAs *in vivo*. *J Cell Biol* **151**: 1–14

Canela N, Rodriguez-Vilarrupla A, Estanyol JM, Diaz C, Pujol MJ, Agell N, Bachs O (2003) The SET protein regulates G2/M transition by modulating cyclin B-cyclin-dependent kinase 1 activity. *J Biol Chem* **278**: 1158–1164

- Costanzo RV, Vila-Ortiz GJ, Perandones C, Carminatti H, Matilla A, Radrizzani M (2006) Anp32e/Cpd1 regulates protein phosphatase 2A activity at synapses during synaptogenesis. *Eur J Neurosci* **23**: 309–324
- del Pozo MA, Kiosses WB, Alderson NB, Meller N, Hahn KM, Schwartz MA (2002) Integrins regulate GTP-Rac localized effector interactions through dissociation of Rho-GDI. *Nat Cell Biol* **4**: 232–239
- del Pozo MA, Price LS, Alderson NB, Ren XD, Schwartz MA (2000) Adhesion to the extracellular matrix regulates the coupling of the small GTPase Rac to its effector PAK. *EMBO J* **19**: 2008–2014
- Fan Z, Beresford PJ, Oh DY, Zhang D, Lieberman J (2003) Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. *Cell* **112**: 659–672
- Grizot S, Faure J, Fieschi F, Vignais PV, Dagher MC, Pebay-Peyroula E (2001) Crystal structure of the Rac1–RhoGDI complex involved in NADPH oxidase activation. *Biochemistry* **40**: 10007–10013
- Haeusler LC, Blumenstein L, Stege P, Dvorsky R, Ahmadian MR (2003) Comparative functional analysis of the Rac GTPases. *FEBS Lett* **555**: 556–560
- Hall A (1998) Rho GTPases and the actin cytoskeleton. *Science* **279**: 509–514
- Heim MH (1999) The Jak–STAT pathway: cytokine signalling from the receptor to the nucleus. *J Recept Signal Transduct Res* **19**: 75–120
- Ho A, Schwarze SR, Mermelstein SJ, Waksman G, Dowdy SF (2001) Synthetic protein transduction domains: enhanced transduction potential *in vitro* and *in vivo*. *Cancer Res* **61**: 474–477
- Jordens I, Marsman M, Kuijl C, Neefjes J (2005) Rab proteins, connecting transport and vesicle fusion. *Traffic* **6**: 1070–1077
- Kahn RA, Volpicelli-Daley L, Bowzard B, Shrivastava-Ranjan P, Li Y, Zhou C, Cunningham L (2005) Arf family GTPases: roles in membrane traffic and microtubule dynamics. *Biochem Soc Trans* **33**: 1269–1272
- Klooster JP, Jaffer ZM, Chernoff J, Hordijk PL (2006) Targeting and activation of Rac1 are mediated by the exchange factor {beta}-Pix. *J Cell Biol* **172**: 759–769
- Kobayashi K, Kuroda S, Fukata M, Nakamura T, Nagase T, Nomura N, Matsuura Y, Yoshida-Kubomura N, Iwamatsu A, Kaibuchi K (1998) p140Sra-1 (specifically Rac1-associated protein) is a novel specific target for Rac1 small GTPase. *J Biol Chem* **273**: 291–295
- Kumar RN, Radhakrishnan R, Ha JH, Dhanasekaran N (2004) Proteome analysis of NIH3T3 cells transformed by activated Galpha12: regulation of leukemia-associated protein SET. *J Proteome Res* **3**: 1177–1183
- Kyosseva SV (2004) Mitogen-activated protein kinase signaling. *Int Rev Neurobiol* **59**: 201–220
- Lechward K, Awotunde OS, Swiatek W, Muszynska G (2001) Protein phosphatase 2A: variety of forms and diversity of functions. *Acta Biochim Pol* **48**: 921–933
- Li M, Guo H, Damuni Z (1995) Purification and characterization of two potent heat-stable protein inhibitors of protein phosphatase 2A from bovine kidney. *Biochemistry* **34**: 1988–1996
- Li M, Makkinje A, Damuni Z (1996) The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J Biol Chem* **271**: 11059–11062
- Madeira A, Pomet JM, Prochiantz A, Allinquant B (2005) SET protein (TAF1beta, I2PP2A) is involved in neuronal apoptosis induced by an amyloid precursor protein cytoplasmic subdomain. *FASEB J* **19**: 1905–1907
- Michaelson D, Silletti J, Murphy G, D'Eustachio P, Rush M, Philips MR (2001) Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding. *J Cell Biol* **152**: 111–126
- Michiels F, Habets GG, Stam JC, van der Kammen RA, Collard JG (1995) A role for Rac in Tiam1-induced membrane ruffling and invasion. *Nature* **375**: 338–340
- Miyaji-Yamaguchi M, Okuwaki M, Nagata K (1999) Coiled-coil structure-mediated dimerization of template activating factor-I is critical for its chromatin remodeling activity. *J Mol Biol* **290**: 547–557
- Miyamoto S, Suzuki T, Muto S, Aizawa K, Kimura A, Mizuno Y, Nagino T, Imai Y, Adachi N, Horikoshi M, Nagai R (2003) Positive and negative regulation of the cardiovascular transcription factor KLF5 by p300 and the oncogenic regulator SET through interaction and acetylation on the DNA-binding domain. *Mol Cell Biol* **23**: 8528–8541
- Olofsson B (1999) Rho guanine dissociation inhibitors: pivotal molecules in cellular signalling. *Cell Signal* **11**: 545–554
- Oxford G, Theodorescu D (2003) Ras superfamily monomeric G proteins in carcinoma cell motility. *Cancer Lett* **189**: 117–128
- Price LS, Langeslag M, ten Klooster JP, Hordijk PL, Jalink K, Collard JG (2003) Calcium signaling regulates translocation and activation of Rac. *J Biol Chem* **278**: 39413–39421
- Price LS, Leng J, Schwartz MA, Bokoch GM (1998) Activation of Rac and Cdc42 by integrins mediates cell spreading. *Mol Biol Cell* **9**: 1863–1871
- Prieto-Sanchez RM, Bustelo XR (2003) Structural basis for the signaling specificity of RhoG and Rac1 GTPases. *J Biol Chem* **278**: 37916–37925
- Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, Horwitz AR (2003) Cell migration: integrating signals from front to back. *Science* **302**: 1704–1709
- Rossman KL, Der CJ, Sondek J (2005) GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol* **6**: 167–180
- Santa-Coloma TA (2003) Anp32e (Cpd1) and related protein phosphatase 2 inhibitors. *Cerebellum* **2**: 310–320
- Seo SB, McNamara P, Heo S, Turner A, Lane WS, Chakravarti D (2001) Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein. *Cell* **104**: 119–130
- Sundaresan P, Farndale RW (2002) P38 mitogen-activated protein kinase dephosphorylation is regulated by protein phosphatase 2A in human platelets activated by collagen. *FEBS Lett* **528**: 139–144
- Tanimukai H, Grundke-Iqbal I, Iqbal K (2005) Up-regulation of inhibitors of protein phosphatase-2A in Alzheimer's disease. *Am J Pathol* **166**: 1761–1771
- Tolias KF, Cantley LC, Carpenter CL (1995) Rho family GTPases bind to phosphoinositide kinases. *J Biol Chem* **270**: 17656–17659
- van Hennik PB, ten Klooster JP, Halstead JR, Voermans C, Anthony EC, Divecha N, Hordijk PL (2003) The C-terminal domain of Rac1 contains two motifs that control targeting and signaling specificity. *J Biol Chem* **278**: 39166–39175
- von Lindern M, van Baal S, Wiegant J, Raap A, Hagemeijer A, Grosveld G (1992) Can, a putative oncogene associated with myeloid leukemogenesis, may be activated by fusion of its 3' half to different genes: characterization of the set gene. *Mol Cell Biol* **12**: 3346–3355
- Wang JG, Collinge M, Ramgolam V, Ayalon O, Fan XC, Pardi R, Bender JR (2006) LFA-1-dependent HuR nuclear export and cytokine mRNA stabilization in T cell activation. *J Immunol* **176**: 2105–2113
- Westphal RS, Coffee Jr RL, Marotta A, Pelech SL, Wadzinski BE (1999) Identification of kinase-phosphatase signaling modules composed of p70 S6 kinase-protein phosphatase 2A (PP2A) and p21-activated kinase-PP2A. *J Biol Chem* **274**: 687–692
- Zhan Q, Ge Q, Ohira T, Van Dyke T, Badwey JA (2003) p21-activated kinase 2 in neutrophils can be regulated by phosphorylation at multiple sites and by a variety of protein phosphatases. *J Immunol* **171**: 3785–3793
- Zhang S, Han J, Sells MA, Chernoff J, Knaus UG, Ulevitch RJ, Bokoch GM (1995) Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. *J Biol Chem* **270**: 23934–23936